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(54) Title: PHOSPHOLIPASE VARIANTS

(57) Abstract: The inventors have used protein engineering to develop variants of fungal phospholipases. Starting from a parent phospholipase, they have modified the amino acid sequence to arrive at variants which have phospholipase activity (generally, at roughly the same level as the parent enzyme) and have a lower lipase activity on triglycerides than the parent enzyme.

WO 2004/111216 A2

PHOSPHOLIPASE VARIANTS

FIELD OF INVENTION

The present invention relates to a method of producing a polypeptide by modifying the amino acid sequence of a polypeptide with phospholipase activity, to a polypeptide having phospholipase activity, and to use of the polypeptide in cheese-making.

BACKGROUND OF THE INVENTION

Lipolytic enzymes are polypeptides with hydrolytic activity for carboxylic ester bonds, e.g., lipase and/or phospholipase activity. The substrate specificity (relative activity on different ester bonds) is important for the usefulness of the lipolytic enzyme in various industrial applications.

WO 00/32758 discloses lipolytic enzyme variants having altered substrate specificity. WO 98/26057 discloses a *Fusarium oxysporum* phospholipase. WO 01/83770 describes lipase variants. WO 00/54601 describes a process for producing cheese from cheese milk treated with a phospholipase.

SUMMARY OF THE INVENTION

The inventors have found that when a fungal phospholipase is used in a cheese-making process, too high lipase activity on triglycerides may lead to a cheese product having changed properties in terms of smell and taste, possibly due to the generation of too many free fatty acids.

To overcome this, the inventors have used protein engineering to develop variants of fungal phospholipases. Starting from a parent phospholipase, they have modified the amino acid sequence to arrive at variants which have phospholipase activity (generally, at roughly the same level as the parent enzyme) and have a lower lipase activity on triglycerides than the parent enzyme. Thus, starting from a parent fungal phospholipase (a polypeptide with phospholipase activity), the inventors have found that the ratio of lipase/phospholipase activity can be decreased by substituting a particular amino acid residue.

The variants are useful in the production of cheese, e.g. in a process or method as described in WO 00/54601, and they result in an increased yield and at the same time avoid the changes in taste and smell, which may result from the generation of too many free fatty acids.

Accordingly, the invention provides a polypeptide which:

a) has phospholipase activity,

b) has an amino acid sequence which is at least 50 % identical to SEQ ID NO: 1, and

c) has one or more of the following amino acids at a position corresponding to SEQ ID NO: 1: D62Q/E/F/W/N/P/L/G; V60R/S/K; S85Y/T; G91R/E; R125K; V203T; V228A; T231R; N233R; L259R/V/P; a deletion D266*; and/or L269A.

The invention also provides a method of producing a polypeptide, comprising:

a) selecting a first (parent) polypeptide which has phospholipase activity and has an amino acid sequence which is at least 50 % identical to SEQ NO: 1,

b) modifying the amino acid sequence by substituting one or more amino acids at a position corresponding to SEQ ID NO: 1: D62Q/E/F/W/N/P/L/G; V60R/S/K; S85Y/T; G91R/E; V203T; V228A; T231R; N233R; L259R/V/P; a deletion D266*; and/or L269A, and

c) preparing a second (modified) polypeptide having the modified amino acid sequence.

The parent polypeptide may also have lipase activity, and the method may further comprise testing the lipase and phospholipase activities of the two polypeptides and selecting a modified polypeptide having a lower lipase/phospholipase ratio than the parent polypeptide.

Further, the invention provides a polynucleotide encoding the polypeptide and a method for producing cheese, comprising the steps of:

a) treating cheese milk or a fraction of the cheese milk with the polypeptide; and

b) producing cheese from the cheese milk during or after step a).

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows an alignment of amino acid sequences of known fungal lipolytic enzymes SEQ ID NO: 1 to 14, as follows:

1: *Thermomyces lanuginosus* (SWISSPROT O59952)

2: *Fusarium oxysporum* (US 6,103,505 SEQ ID NO: 2, GENESEQP AAW51767)

3: *Absidia reflexa* (US 5,821,102 SEQ ID # 10, GENESEQP AAW77403)

4: *Absidia corymbifera* (US 5,821,102 SEQ ID # 6, GENESEQP AAW26689)

5: *Rhizomucor miehei* (SWISSPROT P19515)

6: *Rhizopus oryzae* (SWISSPROT P21811)

7: *Aspergillus niger* (SWISSPROT O42807)

8: *Aspergillus tubingensis* (SWISSPROT O42815)

9: *Fusarium heterosporum* (TREMBL Q02351)

10: *Aspergillus oryzae* (TREMBL P78583)

11: *Penicillium camemberti* (SWISSPROT P25234)

12: *Aspergillus foetidus* (US 5,965,422 SEQ ID # 2, GENESEQP AAW33009)

13: *Aspergillus niger* (WO 98/31790 SEQ ID # 2, GENESEQP AAW64449)

14: *Aspergillus oryzae* (JP 10-155493 SEQ ID # 2, GENESEQP AAW 58541)

DETAILED DESCRIPTION OF THE INVENTION

Parent polypeptide

The polypeptide of the invention may be derived from a parent polypeptide with phospholipase activity, particularly a phospholipase A1, classified as EC 3.1.1.32 according to Enzyme Nomenclature (available at http://www.chem.qmw.ac.uk/iubmb/enzyme). It may be a naturally occurring fungal enzyme with phospholipase activity, e.g. one of SEQ ID NO: 2-14, particularly a phospholipase from *Fusarium oxysporum* which is described in WO 98/26057. Alternatively, the parent may be a fungal lipolytic enzyme variant with phospholipase activity as disclosed in WO 00/32758, e.g. a variant of SEQ ID NO: 1 as described in Example 5 of WO 00/32758.

Lipase and phospholipase activities

Lipase activity is measured by the SLU method described in WO 0032758, and the lipase activity of the pure protein is expressed as SLU per unit of A280 (Absorption at 280 nm).

Phospholipase activity is measured by incubating 0.025-0.07 mg enzyme protein (e.g. 0.05 mg) with cream (standardized to 25 % fat by mixing with skimmed milk) at 35 C for 1.5 hr without shaking and measuring phospholipid depletion (by lipid extraction and HPLC analysis). Phospholipase activity is expressed as % PL depletion.

The variant polypeptides of the invention typically show 15-75 % PL depletion by this method. The lipase activity is typically below 1000 SLU/A280, particularly below 500, below 250, below 100 or below 25. The PL/lipase ratio is typically above 0.05, particularly above 0.1, above 0.2, above 0.3, above 1, above 2 or above 3.

The phospholipase activity can also be determined by known methods, e.g. as described in WO 0032758, by HPLC or by phospholipid depletion in cream. Using the "monolayer phospholipase assay" described in WO 0032758, the parent and the modified polypeptide may have a phospholipase activity of at least 0.25 nmol/min at enzyme dose 60 µg and 25°C; e.g. at least 0.40 nmol/min, at least 0.75 nmol/min, at least 1.0 nmol/min, at least 1.25 nmol/min, or at least 1.5 nmol/min.

Amino acid alteration

The modified polypeptide has one or more of the following amino acids at a position corresponding to the following in SEQ ID NO: 1: D62Q/E/F/W/V/P/L/G; V60R/S/K; R84G/S; S85Y/T; G91R/E; R125K; V203T; V228A; T231R; N233R; L259R/V/P; a deletion D266*; and/or L269A. Corresponding positions in SEQ ID NO: 2-14 are defined by the alignment shown in Figure 1, e.g. position I83 of SEQ ID NO: 2. Corresponding positions in other sequences may be found by an alignment as described below.

Compared to SEQ ID NO: 1, the polypeptide of the invention may further have one or more of the following amino acids at a position corresponding to the following in SEQ ID NO: 1: D57G, V60G/C/K/R/L/S/Q, D62H/A, S83T, R84G/S/W; G91A/V, L93K, D96W/F/G, E99K, R125K, L259S, F262L, G263Q, L264A, I265T, G266D, T267A/E and/or L269N. Also, N- and/or C-terminus may be extended, e.g. as described in WO 9704079. Thus, the C-terminal may be extended by adding residues after position 269, e.g. addition of AGGFS or AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS. The N-terminal may br extended by the addition of amino acid residues such as SPIRR. Such C- or N-terminal extensions should not be considered, when calculating the amino acid identity with SEQ ID NO: 1.

Sequences derived from SEQ ID NO: 2 may be C-terminal processed (e.g. during expression in *A. oryzae)*, e.g. with positions 272, 273, 274 or 286 of SEQ ID NO 2 as the C-terminal residue.

The parent and modified polypeptides may be tested for lipase and phospholipase activity, and a variant polypeptide may be selected which has phospholipase activity and a lipase/phospholipase ratio which is lower than the parent polypeptide. Lipase activity can be determined by known methods using a triglyceride as substrate, e.g. as described in WO 00/32758.

Amino acid identity and alignment

The amino acid identity may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

The variant polypeptide has an amino acid identity to SEQ ID NO: 1 which is at least 50%, particularly at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for

polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

EXAMPLES

Example 1. Construction of variants having a increased phospholipase/lipase activity ratio compared to the parent enzyme.

The following variant polypeptides were constructed as described in WO 00/32758. Each polypeptide is described by the amino acid alterations compared to SEQ ID NO: 1.

Variant	Amino acid alteration in SEQ ID NO: 1
1	R84W +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
2	R84W +G91E +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
3	V60G +D62E +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
5	R84W +G91R +L93K +D96G +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
6	V60G +D62F +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
7	R84W +S85Y +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
8	R84W +G91A +D96W +E99K +L259V +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
10	V60G +D62W +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
11	R84W +G91R +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS

12	V6OC +D62H +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
13	V60G +D62V +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
14	V60K +D62L +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
15	V60R +D62L +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
16	V60G +D62G +R84W +G91A +D96W +V228A +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
17	V60L +D62A +R84W +G91A +D96W +E99K +R125K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
18	D62E +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
19	V60S +D62L +R84W +G91A +D96F +E99K +F262L +G263Q +L264A +I265T +G266D +T267A +L269N
20	D57G +V60Q +D62P +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
21	R84W +G91A +D96W +E99K +L259R +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
23	D62Q +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
25	R84W +G91A +D96W +E99K +V203T +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
26	R84S +S85T +G91A +D96S +T231R +N233R +L259P +G263Q +L264S +I265T +G266* +T267E +L269A

Each of the above variant polypeptides showed a phospholipase depletion of 15-75 %, a lipase activity below 250 SLU/A280 and a PL/lipase activity above 0.1. For comparison, a

number of prior-art variants described in Example 5 of WO 0032758 were measured and were found to have a PL/lipase ratio below 0.05.

Example 2. Evaluation of cheese yield using selected variants of the invention

The following variant polypeptides from Example 1 were evaluated in a method of producing cheese with the addition of a phospholipase. The controls were without phospholipase addition.

The method was a bench top cheese yield evaluation test and was performed as described below.

1. Standardize 0.5 kg cheese milk w/ pasteurized skim milk and cream.

2. Prepare a single starter by adding 0.1 g Rhodia LH100 and 0.3 g Rhodia TA061 starter cultures (for mozzarella) to 50 ml of the skim milk and equilibrate to 35°C w/ gentle, continuous stirring.

3. Equilibrate cheese milk to 35°C and add 0.07 mg enzyme protein per g fat, check initial pH and add 5 ml starter to each cheese milk with gentle agitation .

4. When pH reaches 6.45 – 6.50 add 0.5 ml of rennet (10x diluted Chymax, available from Christian Hansen); stir vigorously for three minutes then remove stirrers from milk, cover water bath and allow milk to coagulate.

5. Cut curd at the appropriate time (30-45 minutes) wit 25 mm (½") knives. To determine cutting time, make a downward cut into the curd with knife or spatula. The curd is ready for cutting when the cut separates upon lifting and sharp edges are maintained on the top surface at the edge of the cut.. Allow the curd to rest for 5 minutes then gently and intermittently stir curd to prevent coalescence of curd particles.

6. Increase temperature to 41°C and hold until curd pH reaches 5.65 – 5.70, then drain and pour curd particles into stainless steel bowls. Float bowls in 41°C water bath to maintain curd temperature. Periodically drain excess whey, leaving only enough to cover curds for maintenance of heat.

7. When curd pH ~ 5.25 - 5.3, drain all whey and flood curd w/ D.I. water at 57°C for 5 min. Stretch the curd by hand for ~ 1min in 59°C water, then place the curd in ice water for 15 min and dry blot. Record weight of curd and refrigerate until further analysis.

Results

Variants No. 2, 4, 5, 8, 9, 10, 16, 22 and 24 of Example 1 were tested. All the tested variants resulted in improved yield compared to the control, when calculated as moisture adjusted yield.

CLAIMS

1. A polypeptide which:

a) has phospholipase activity,

b) has an amino acid sequence which is at least 50 % identical to SEQ ID NO: 1, and

c) has one or more of the following amino acids at a position corresponding to SEQ ID NO: 1: D62Q/E/F/W/V/P/L/G; V60R/S/K; S85Y/T; G91R/E; R125K; V203T; V228A; T231R; N233R; L259R/V/P; a deletion D266*; and/or L269A.

2. The polypeptide of claim 1, which has one or more of the following amino acids at a position corresponding to SEQ ID NO: 1: D57G, V60G/C/L/Q, D62H/A, S83T, R84G/S/W; G91A/V, L93K, D96W/F/G, E99K, R125K, L259S, F262L, G263Q, L264A, I265T, G266D, T267A/E and/or L269N and/or by a C-terminal extension, particularly AGGFS or AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS.

3. The polypeptide of claim 1 or 2 which has the sequence of SEQ ID NO: 1 with one of the following sets of alterations:

R84W +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R84W +G91E +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V60G +D62E +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
R84W +G91R +L93K +D96G +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V60G +D62F +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R84W +S85Y +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R84W +G91A +D96W +E99K +L259V +G263Q +L264A +I265T +G266D +T267A +L269N

+270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V60G +D62W +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
R84W +G91R +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V6OC +D62H +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
V60G +D62V +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
V60K +D62L +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
V60R +D62L +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
V60G +D62G +R84W +G91A +D96W +V228A +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V60L +D62A +R84W +G91A +D96W +E99K +R125K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
D62E +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
V60S +D62L +R84W +G91A +D96F +E99K +F262L +G263Q +L264A +I265T +G266D +T267A +L269N
D57G +V60Q +D62P +R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N
R84W +G91A +D96W +E99K +L259R +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
D62Q +R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R84W +G91A +D96W +E99K +V203T +G263Q +L264A +I265T +G266D +T267A +L269N

+270A +271G +272G +273F +274S +275WRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R84S +S85T +G91A +D96S +T231R +N233R +L259P +G263Q +L264S +I265T +G266* +T267E +L269A

4. A polynucleotide encoding the polypeptide of any of claims 1-3.

5. A method of producing a polypeptide, comprising:

a) selecting a first polypeptide which has phospholipase activity and has an amino acid sequence which is at least 50 % identical to SEQ NO: 1,

b) altering the amino acid sequence wherein the alteration comprises one or more substitutions or deletion corresponding to the following in SEQ ID NO: 1: D62Q/E/F/W/V/P/L/G; V60R/S/K; S85Y/T; G91R/E; V203T; V228A; T231R; N233R; L259R/V/P; a deletion D266*; and/or L269A, and

c) preparing a second polypeptide having the modified amino acid sequence.

6. The method of claim 5 whereln the selected polypeptide has lipase activity, and the method further comprises testing the lipase and phospholipase activities of the two polypeptides and selecting a second polypeptide having a lower lipase/phospholipase ratio than the first polypeptide.

7. A method for producing cheese, comprising the steps of:

a) treating cheese milk or a fraction of the cheese milk with the polypeptide of any of claims 1-3 or a polypeptide produced by the method of claim 5 or 6; and

b) producing cheese from the cheese milk during or after step a).

Figure 1.

Alignment of fungal lipolytic enzyme sequences

```
                    1                                                  50
SEQ ID NO: 1    .......... EVSQDLFNQF NLFAQYSAAA .......YCG KNNDAPAGTN    33
SEQ ID NO: 2    .........AV GVTTTDFSNF KFYIQHGAAA .......YC. .NSEAAAGSK    33
SEQ ID NO: 3    SSSSTQDYRI ASEAEIKAHT FYTALSANA. .......YCR TVIPG.....
SEQ ID NO: 4    .SSSTQDYRI ASEAEIKAHT FYTALSANA. .......YCR TVIPG.....
SEQ ID NO: 5    ..SIDGGIRA ATSQEINELT YYTTLSANS. .......YCR TVIPG.....
SEQ ID NO: 6    .SASDGGKVV AATTAQIQEF TKYAGIAATA .......YCR SVVPG.....
SEQ ID NO: 7    ....TAGQAL AASTQ.GISE DLYNRL.VEM ATISQAAYAD LCNIPST...
SEQ ID NO: 8    ....TAGHAL AASTQ.GISE DLYSRL.VEM ATISQAAYAD LCNIPST...
SEQ ID NO: 9    .......... TVTTQDLSNF RFYLQHADAA .......YC. .NFNTAVGKP
SEQ ID NO: 10   .......... DIPTTQLEDF KFWVQYAAAT .......YCP NNYVAKDGEK
SEQ ID NO: 11   .......... DVSTSELDQF EFWVQYAAAS .......YYE ADYTAQVGDK
SEQ ID NO: 12   .......... SVSTSTLDEL QLFAQWSAAA .......YCS NNID.SKDSN
SEQ ID NO: 13   .......... SVSTSTLDEL QLFSQWSAAA .......YCS NNID.SDDSN
SEQ ID NO: 14   .......... DVSSSLLNNL DLFAQYSAAA .......YCD ENLN.STGTK

                   51                                                 100
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SEQ ID NO: 2    ITCSNNGCPT VQGNGATIVT SF..VGSKTG IGGYVATDSA RKEIVVSFRG    81
SEQ ID NO: 3    GRWSCPHCGV AS..NLQITK TFST..LITD TNVLVAVGEK EKTIYVVFRG
SEQ ID NO: 4    GQWSCPHCDV AP..NLNITK TFTT..LITD TNVLVAVGEN EKTIYVVFRG
SEQ ID NO: 5    ATWDCIHCDA TE..DLKIIK TWST..LIYD TNAMVARGDS EKTIYIVFRG
SEQ ID NO: 6    NKWDCVQCQK WVP.DGKIIT TFTS..LLSD TNGYVLRSDK QKTIYLVFRG
SEQ ID NO: 7    .......... .......IIK GEKIYNAQTD INGWILRDDT SKEIITVFRG
SEQ ID NO: 8    .......... .......IIK GEKIYNSQTD INGWILRDDS SKEIITVFRG
SEQ ID NO: 9    VHCSAGNCPD IEKDAAIVVG SV..VGTKTG IGAYVATDNA RKEIVVSVRG
SEQ ID NO: 10   LNCSVGNCPD VEAAGSTVKL SFS.DDTITD TAGFVAVDNT NKAIVVAFRG
SEQ ID NO: 11   LSCSKGNCPE VEATGATVSY DFS.DSTITD TAGYIAVDHT NSAVVLAFRG
SEQ ID NO: 12   LTCTANACPS VEEASTTMLL EFDLTNDFGG TAGFLAADNT NKRLVVAFRG
SEQ ID NO: 13   VTCTADACPS VEEASTKMLL EFDLTNNFGG TAGFLAADNT NKRLVVAFRG
SEQ ID NO: 14   LTCSVGNCPL VEAASTQSLD EFNESSSYGN PAGYLAADET NKLLVLSFRG

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SEQ ID NO: 2    SINIRNWLTN LDFG.QEDCS L..VSGCGVH SGFQRAWNEI SSQATAAVAS   128
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SEQ ID NO: 4    TSSIRNAIAD IVFVPVNYPP V...NGAKVH KGFLDSYNEV QDKLVAEVKA
SEQ ID NO: 5    SSSIRNWIAD LTFVPVSYPP V...SGTKVH KGFLDSYGEV QNELVATVLD
SEQ ID NO: 6    TNSFRSAITD IVFNFSDYKP V...KGAKVH AGFLSSYEQV VNDYFPVVQE
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SEQ ID NO: 8    TGSDTNLQLD TNYTLTPFDT LPQCNSCEVH GGYYIGWISV QDQVESLVQQ
SEQ ID NO: 9    SINVRNWITN FNFG.QKTCD L..VAGCGVH TGFLDAWEEV AANVKAAVSA
SEQ ID NO: 10   SYSIRNWVTD ATFP.QTDPG L..CDGCKAE LGFWTAWKVV RDRIIKTLDE
SEQ ID NO: 11   SYSVRNWVAD ATFV.HTNPG L..CDGCLAE LGFWSSWKLV RDDIIKELKE
SEQ ID NO: 12   SSTIENWIAN LDFILEDNDD L..CTGCKVH TGFWKAWESA ADELTSKIKS
SEQ ID NO: 13   SSTIKNWIAD LDFILQDNDD L..CTGCKVH TGFWKAWEAA ADNLTSKIKS
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SEQ ID NO: 4    QLDRHPGYKI VVTGHSLGGA TAVLSALDLY HHGHD....N IEIYTQGQPR
SEQ ID NO: 5    QFKQYPSYKV AVTGHSLGGA TALLCALDLY QREEGLSSSN LFLYTQGQPR
SEQ ID NO: 6    QLTAHPTYKV IVTGHSLGGA QALLAGMDLY QREPRLSPKN LSIFTVGGPR
SEQ ID NO: 7    QASQYPDYAL TVTGHSLGAS MAALTAAQL. SATYD....N VRLYTFGEPR
SEQ ID NO: 8    QVSQFPDYAL TVTGHSLGAS LAALTAAQL. SATYD....N IRLYTFGEPR
SEQ ID NO: 9    AKTANPTFKF VVTGHSLGGA VATIAAAYLR KDGF.....P FDLYTYGSPR
SEQ ID NO: 10   LKPEHSDYKI VVVGHSLGAA IASLAAADLR TKNY.....D AILYAYAAPR
```

Fig. 1 cont.

```
SEQ ID NO: 11   VVAQNPNYEL VVVGHSLGAA VATLAATDLR GKGYP....S AKLYAYASPR
SEQ ID NO: 12   AMSTYSGYTL YFTGHSLGGA LATLGATVLR NDGY.....S VELYTYGCPR
SEQ ID NO: 13   AMSTYSGYTL YFTGHSLGGA LATLGATVLR NDGY.....S VELYTYGCPR
SEQ ID NO: 14   ALSDHSDYSL VLTGHSYGAA LAALAATALR NSGH.....S VELYNYGQPR

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SEQ ID NO: 4    IGTPEFANYV IGT..... KIPYQRLVNE RDIVPHLPPG AFGFLHAGEE
SEQ ID NO: 5    VGDPAFANYV VST..... GIPYRRTVNE RDIVPHLPPA AFGFLHAGEE
SEQ ID NO: 6    VGNPTFAYYV EST..... GIPFQRTVHK RDIVPHVPPQ SFGFLHPGVE
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SEQ ID NO: 8    S.NQAFASYM NDAFQASSPD TTQYFRVTHA NDGIPNLPPA DEGYAHGVVE
SEQ ID NO: 9    VGNDFFANFV TQQ..... TGAEYRVTHG DDPVPRLPPI VFGYRHTSPE
SEQ ID NO: 10   VANKPLAEFI TNQ..... .GNNYRFTHN DDPVPKLPLL TMGYVHISPE
SEQ ID NO: 11   VGNAALAKYI TAQ..... .GNNFRFTHT NDPVPKLPLL SMGYVHVSPE
SEQ ID NO: 12   IGNYALAEHI TSQ......G SGANFRVTHL NDIVPRVPPM DFGFSQPSPE
SEQ ID NO: 13   VGNYALAEHI TSQ......G SGANFPVTHL NDIVPRVPPM DFGFSQPSPE
SEQ ID NO: 14   LGNEALATYI TDQ......N KGGNYRVTHT NDIVPKLPPT LLGYHHFSPE

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SEQ ID NO: 3    FWIMK..... ....DSSLRV CPNGIETDNC SNSIVPFT.. SVIDHLSYLD
SEQ ID NO: 4    FWIMK..... ....DSSLRV CPNGIETDNC SNSIVPFT.. SVIDHLSYLD
SEQ ID NO: 5    YWITD..... ..NSPETVQV CTSDLETSDC SNSIVPFT.. SVLDHLSYFG
SEQ ID NO: 6    SWIKS..... ...GTSNVQI CTSEIETKDC SNSIVPFT.. SILDHLSYFD
SEQ ID NO: 7    YWSV....DP YSAQNTFVCT GDEVQCCE.A QGGQGVN... ..DAHTTYF.
SEQ ID NO: 8    YWSV....DP YSAQNTFVCT GDEVQCCE.A QGGQGVN... ..NAHTTYP.
SEQ ID NO: 9    YWLNG.GPLD KDYTVTEIKV CEGIANVM.C NGGTIGL... DILAHITYF.
SEQ ID NO: 10   YYITA..PDN TTVTDNQVTV LDGYVNFK.G NTGTSGGLPD LLAFHSHVWY
SEQ ID NO: 11   YWITS..PNN ATVSTSDIKV IDGDVSFD.G NTGTGLPLLT DFEAHIWYF.
SEQ ID NO: 12   YWITS..GNG ASVTASDIEV IEGINSTA.G NAGEATV... SVLAHLWYF.
SEQ ID NO: 13   YWITS..GTG ASVTASDIEL IEGINSTA.G NAGEATV... DVLAHLWYF.
SEQ ID NO: 14   YYISS..ADE ATVTTTDVTE VTGIDATG.G NDGTDGT... SIDAHRWYF.

                301                                                 350
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<120> Lipolytic enzyme variants

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180 185 190

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115 120 125

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Tyr Phe Arg Val Thr His Ser Asn Asp Gly Ile Pro Asn Leu Pro Pro
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210 215 220

Pro Tyr Ser Ala Gln Asn Thr Phe Val Cys Thr Gly Asp Glu Val Gln
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Tyr Asn Ser Gln Thr Asp Ile Asn Gly Trp Ile Leu Arg Asp Asp Ser
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Ser Lys Glu Ile Ile Thr Val Phe Arg Gly Thr Gly Ser Asp Thr Asn
65 70 75 80

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85 90 95

Gln Cys Asn Ser Cys Glu Val His Gly Gly Tyr Tyr Ile Gly Trp Ile
100 105 110

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115 120 125

Phe Pro Asp Tyr Ala Leu Thr Val Thr Gly His Ser Leu Gly Ala Ser
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Leu Ala Ala Leu Thr Ala Ala Gln Leu Ser Ala Thr Tyr Asp Asn Ile
145 150 155 160

Arg Leu Tyr Thr Phe Gly Glu Pro Arg Ser Asn Gln Ala Phe Ala Ser
165 170 175

Tyr Met Asn Asp Ala Phe Gln Ala Ser Ser Pro Asp Thr Thr Gln Tyr
180 185 190

Phe Arg Val Thr His Ala Asn Asp Gly Ile Pro Asn Leu Pro Pro Ala
195 200 205

Asp Glu Gly Tyr Ala His Gly Val Val Glu Tyr Trp Ser Val Asp Pro
210 215 220

Tyr Ser Ala Gln Asn Thr Phe Val Cys Thr Gly Asp Glu Val Gln Cys
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Cys Glu Ala Gln Gly Gly Gln Gly Val Asn Asn Ala His Thr Thr Tyr
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Phe Gly Met Thr Ser Gly His Cys Thr Trp
260 265

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35 40 45

Val Val Gly Ser Val Val Gly Thr Lys Thr Gly Ile Gly Ala Tyr Val
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Ile Asp Ala Thr Gly Gly Asn Asp Gly Thr Asp Gly Thr Ser Ile Asp
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Ala His Arg Trp Tyr Phe Ile Tyr Ile Ser Glu Cys Ser
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(19) World Intellectual Property Organization
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23 December 2004 (23.12.2004)

PCT

(10) International Publication Number
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(21) International Application Number: PCT/DK2004/000426

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(30) Priority Data:
60/479,647 19 June 2003 (19.06.2003) US

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(72) Inventors; and

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(74) Common Representative: **NOVOZYMES A/S**; Patents, Drogshøjvej 36, DK-2880 Bagsværd (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
24 February 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PHOSPHOLIPASE VARIANTS

(57) Abstract: The inventors have used protein engineering to develop variants of fungal phospholipases. Starting from a parent phospholipase, they have modified the amino acid sequence to arrive at variants which have phospholipase activity (generally, at roughly the same level as the parent enzyme) and have a lower lipase activity on triglycerides than the parent enzyme.

WO 2004/111216 A3

INTERNATIONAL SEARCH REPORT

International Application No
I 'DK2004/000426

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/20 C12N15/55 C11D3/386 A23C19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, EMBL, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/32758 A (SHAMKANT ANANT PATKAR ; BORCH KIM (DK); PETRI ANDREAS (DK); VIND JESPE) 8 June 2000 (2000-06-08)	1,2,4-6
Y	see claims, especially claims 27 to 29 for invention 2: see page 10, l.7-9, p. 41, p. 46, l. 6, p. 47, l. 6-7, claims 10, 17, 36, 39	2,7
Y	WO 00/54601 A (NOVONORDISK AS) 21 September 2000 (2000-09-21) see the whole document	7
Y	WO 02/055679 A (DANIELSEN STEFFEN ; BORCH KIM (DK); VIND JESPER (DK); MINNING STEFAN () 18 July 2002 (2002-07-18) see claims; for invention 2: see p. 12, l. 10, claim 10 item (kk)	2

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 2004	11. 01. 05

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Grosskopf, R

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
/DK2004/000426

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/83770 A (ROGGEN ERWIN LUDO ; NOVOZYMES AS (DK)) 8 November 2001 (2001-11-08) see claims	2
Y	WO 95/22615 A (THELLERSEN MARIANNE ; NOVONORDISK AS (DK); SVENDSEN ALLAN (DK); CLAUSE) 24 August 1995 (1995-08-24) see claims; for invention 2: see p. 13, l. 20, p. 20, l. 15, p. 74, line 18	2

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/DK2004/000426

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

Claims 1 to 7 (all partially)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position D62 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

2. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position V60 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

3. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position S85 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

4. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position G91 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

5. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position R125 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

6. claims: Claims 1-7 (all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position V203 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

7. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position V228 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

8. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position T231 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

9. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position N233 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

10. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position L259 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

11. claims: Claims 1-7 (all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a deletion at a position which corrresponds to position D266 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

12. claims: Claims 1-7 (all partially)

Polypeptide having phospholipase activity and which has an amino acid sequence which is at least 50% identical to SEQ ID NO: 1 and has a substitution at a position which corrresponds to position L269 in SEQ ID NO:1 , polynucleotide encoding it, methods for preapring it and the use of said polypeptide

---

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.:

On the basis of the Figure 1 shown in the application it may be possible to search mutations in the corresponding positions of the SEQ ID NOs: 2 to 14. However, it is impossible to determine (and consequently to search) the corresponding position in a lipase which is merely characterised by the fact that it is 50% identical to SEQ ID NO:1

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

nformation on patent family members

International Application No
/DK2004/000426

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0032758	A	08-06-2000	AU	1376300 A	19-06-2000
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